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# hNT Neurons Express an Immunosuppressive Protein that Blocks T-lymphocyte Proliferation and Interleukin-2 Production.

I like it. I think it is a bit long.

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**ABSTRACT**

Ntera2/D1 cells had an A1 B8 Bw6 Cw7 DR3 DR52 major histocompatibility complex (MHC) genotype. Its neuronal derivative, hNT neurons, expressed A1 B8 Bw6 MHC class I molecules, but did not activate, and hNT supernatant suppressed allogeneic mixed lymphocyte cultures (MLC) >98% ($p<0.01$). hNT supernatant suppressed phytohemagglutinin (PHA)-activated T-cell proliferation, even 48 hours after stimulation, suppressed phorbol 12-myristate 13-acetate (PMA) and/or ionomycin-induced T-cell proliferation >99% ($p<0.001$), and reduced interleukin-2 (IL-2) production ($p<0.01$), while maintaining T-cells in a quiescent $G_0/G_1$ state without lowering their viability. The immunosuppressive activity of hNT supernatant was attributed to a 40-100 kDa anionic protein with an isoelectric point of 4.8.

Key Words: Neuron, Protein, Immunosuppresion, T-lymphocyte.

## 1. INTRODUCTION

The central nervous system (CNS) actively maintains immune privilege (Carson and Sutcliffe, 1999; Fabry et al., 1994), in part by restricting immune cell access (Goldstein and Betz, 1986; Hickey et al., 1991), limiting afferent antigen drainage (Weller et al., 1996; Cserr and Knopf, 1992), suppressing immune responsiveness (Irani et al., 1996; Irani et al., 1997), guiding the recruitment and differentiation of effector cell phenotypes (Aloisi et al., 1998; Carson et al., 1999), and possessing weak antigen presenting cells (Carson et al., 1998).

Neurons may directly modulate immune responsiveness. Absence of constitutive neuronal major histocompatibility complex (MHC) expression may limit anti-neuronal cytotoxic T-cell effector mechanisms (Rall, 1998). Glycosphingolipids known as gangliosides are enriched within neurons, can be shed from the cell surface, are immunosuppressive, and may contribute to immune privilege (Irani et al., 1996; Rall, 1998). Gangliosides suppress the expression of MHC molecules (Massa, 1993), the proliferation of T-cells, and the production of interleukin-2 (IL-2) (Irani et al., 1996; Irani et al., 1997; Bergelson, 1995; Robb, 1986; Dyatlovitskaya and Bergelson, 1987).

The therapeutic approach of transfecting and transplanting neurons to ameliorate neurological deficits requires a defined, preferably clonal source of differentiated human neurons amendable to efficient transfection and sustained expression of therapeutic genes (Trojanowski et al., 1997; Cook et al., 1994). A therapeutic effect is anticipated should the engrafted cells retain a neuronal phenotype, functionally integrate, and deliver a sustained level of therapeutically relevant protein to the affected region of the brain (Cook et al., 1994). This approach has evolved from trials utilizing neuronal isolates of the embryonic ventral mesencephalon (Kordower et al., 1995; Bjorklund, 1992; Perlow et al., 1979), or modified neuronal progenitors (Sabate et al., 1995), neurons (Anton et al., 1994), or fibroblasts (Fisher et al., 1991). Ganglioside shedding, and the absence of MHC expression may favor resistance of the neuronal graft to MHC-restricted T-cell attack (Lampson and Siegel, 1988).

Embryonic neurons as grafts are limited by their heterogeneity, expense, scarcity, diminishing viability over time, and by being refractory to standard transfection techniques (Cook et al., 1994; Meichsner et al., 1993). A promising alternative neuron, which is amendable to transfection, is derived from the embryonal carcinoma cell line Ntera2/D1, a putative neuronal progenitor (Cook et al., 1994; Andrews et al., 1984). Ntera2/D1 differentiate in response to treatment with all-*trans*-retinoic acid into a mixture

of cells, including postmitotic cells with a neuronal phenotype (Andrews, 1984; Pleasure et al., 1992; Pleasure and Lee, 1993). Cultures are selectively enriched for Ntera2/D1-derived neurons (designated hNT) by inhibiting non-neuronal cells with mitotic inhibiters, and by replating hNT on poly-D-lysine plus laminin, which encourages growth of polarized processes. In this manner, cultures comprised of >90% hNT neurons are prepared (Cook et al., 1994).

hNT neurons have identifiable axons and dendrites (Andrews, 1984), retain a plasticity to regenerate and extend neurites after multiple replatings *in vitro* (Cook et al., 1994), and express neurofilaments characteristic of neuronal development and the adult central nervous system (Andrews, 1984; Lee and Andrews, 1986). hNT neurons synthesize neurotransmitters, express the catecholamine biosynthetic enzyme tyrosine hydroxylase, and excrete the dopamine metabolite homovanillic acid (Zeller and Strauss, 1995; Iacovitti and Stull, 1997). Transplanted hNT neurons are capable of long-term functional integration (Kleppner et al., 1995), are nontumorigenic (Trojanowski et al., 1997), and can correct behavioral deficits in the lesioned rodent (Borlongan et al.,1998).

A paucity of data exists regarding the MHC and immunological features of hNT neurons. Retinoic acid-induced differentiation of Ntera2/D1 causes the produced hNT neurons to express MHC class I and β-2 microglobulin molecules (Segars et al., 1993), but whether hNT neurons express a discernable MHC phenotype that can activate allogeneic immunocytes has not been determined. An increase in the expression of gangliosides (e.g., $GD_3$ and $GT_3$) and the glycolipid sialyltransferases that contribute to their synthesis occurs during the differentiation of embryonal carcinoma cells (Chen et al., 1989; Osania et al., 1997), but whether hNT neurons shed gangliosides at immunosuppressive levels has not been determined. Some CNS neoplasms (e.g., gliomas) express immunosuppressive levels of transforming growth factor-β (TGF-β) (Weller and Fontana, 1995). TGF-β inhibits T-cell proliferation by suppressing IL-2-mediated proliferative signals (Ahuja et al., 1993). Retinoic acid treatment increases TGF-β expression during murine embryogenesis (Mahmood et al., 1995), and during embryonal carcinoma cell differentiation (Rizzino et al., 1983), but whether hNT neurons express immunosuppressive levels of TGF-β has not been determined.

In light of the therapeutic potential of hNT neuronal grafts, we evaluated hNT for its MHC and immunological characteristics, and for evidence of neuronal regulation of immune cells *in vitro*. During this evaluation, we quite serendipitously discovered a novel hNT neuron-expressed immunosuppressive protein with characteristics unlike

gangliosides or TGF-β, which is potently suppressive of T-cell activation, proliferation, and the production of IL-2. Consequently, hNT neuronal grafts may prove to be both therapeutic and self-protective, engrafted alone, or as co-grafts with other neurons.

## 2. MATERIAL & METHODS:

**Production of Ntera2/D1.** Ntera2/D1 cells (Layton Bioscience, Inc., Gilroy, CA.) were maintained in Dulbecco's minimal essential medium with nutrient mixture F-12 (DMEM/F-12) with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD.), and incubated in a 37°C, humidified, 5% $CO_2$ environment.

**Differentiation to hNT neurons.** Differentiation of Ntera2/D1 is described in detail elsewhere (Andrews, 1984; Pleasure et al., 1992). Briefly, Ntera2/D1 ($2 \times 10^6$) were treated with 10 μM *all-trans* retinoic acid (Sigma, St. Louis, MO.) for 5-6 weeks. Mitotic non-neuronal cells were inhibited with 1μM cytosine β-D-arabinofuranoside, 10μM 5-Fluoro-2'-deoxyuridine, and 10μM 1-β-D-ribofuranosyluracil (Sigma) for 1 week. Some retinoic acid-treated Ntera2/D1 cultures were exposed to 25 Gy γ-irradiation ($^{137}Cs$) to inhibit non-neuronal cells, and were not treated with mitotic inhibitors, to determine whether the immunosuppressive properties of hNT supernatant was attributable to trace amounts of mitotic inhibitors. Differentiated hNT neurons overlying the dense cellular mixture were treated with 0.025% trypsin and 0.01% EDTA, dislodged, and replated at a density of $2\text{-}3 \times 10^6$ cells/ml in serum-free Opti-Mem medium (Gibco-BRL), or DMEM/F-12 10% FBS medium, each supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, and 2.0 mM L-glutamine, in flasks pretreated with poly-D-lysine (Sigma) and coated with MATRIGEL® basement membrane matrix (Collaborative Research/Becton Dickinson, Bedford, MA.). Cultures were fed serum-free Opti-Mem medium or DMEM/F-12 10% FBS medium for up to 6 days, yielding hNT supernatant which was filter sterilized and stored at -20°C for future analysis. Enriched hNT neuronal cultures consisted of >90% neurons.

**MHC Genotype & Phenotype.** Ntera2/D1 genomic DNA (75–125 ng/μl) was used as template for analysis by sequence-specific primer polymerase chain reaction (Bunce et al., 1995) in MHC diagnostic plates (Pel-Freez Clinical Systems, Deerbrook Trail, WI), and the amplified products analyzed by ethidium bromide stained electrophoresis.

hNT MHC surface expression was evaluated using a complement-dependent cytotoxic technique in Terasaki tissue typing trays (One Lambda, Inc., Canoga Park, CA). Briefly, $2 \times 10^6$/ml hNT neurons were reacted with MHC antigen-specific monoclonal antibody (mAb) or known antisera, mixed with rabbit complement, ethidium

bromide and acridine orange, and the reaction stopped by hemoglobin-EDTA. Cell viability was scored, and the MHC phenotype determined. At least 2 mAb or 3 overlapping antisera were used to define each MHC antigen.

**Isolation of PBMC.** Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy human donors by layering over Accu-Prep (Accurate Chemical Corp., Westbury, NY). The interface band was collected, washed, then suspended in either serum-free Opti-Mem medium, or in RPMI-1640 with 10% FBS supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin and 2.0 mM L-glutamine (RPMI medium).

**Mixed Lymphocyte and Mixed Lymphocyte-Neuron Cultures.** Mixed lymphocyte cultures (MLC) consisted of $10^5$ responder and $10^5$ allogeneic stimulator PBMC. Mixed lymphocyte-neuron cultures (MLNC) consisted of $10^5$ responder PBMC and $10^5$ stimulator hNT neurons. MLC or MLNC were established in triplicates, incubated for 4 days, pulsed with $^3$H-thymidine ($^3$H-TdR), harvested (Packard Instrument, Meriden, CT), and counted in a β-spectrometer (Packard Instrument). The uptake of $^3$H-TdR by stimulator cells was prevented by prior 25 Gy γ-irradiation ($^{137}$Cs). The stimulation index of responder PBMC was determined by dividing the mean cpm of triplicate stimulated cultures by the mean cpm of triplicate control syngeneic cultures. Assays were prepared using either serum-free Opti-Mem medium, RPMI medium, or 1:2 hNT supernatant. Viability of PBMC in MLC and MLNC was assured by trypan blue dye exclusion on day 4.

**T-Lymphocyte Proliferation Assays:** The accessory cell-dependent mitogens phytohemagglutinin (PHA) at 1:50 or 1:250, or concanavalin A at 1:20, which cross-link the T-cell receptor and trigger T-lymphocyte proliferation were used to activate triplicate cultures of $10^5$ PBMC. Assays were prepared using either serum-free Opti-Mem medium, RPMI medium, or 1:2 hNT supernatant, incubated for 48 hours, pulsed, harvested, and the uptake of $^3$H-TdR determined. Viability of PHA-stimulated PBMC was assured by trypan blue dye exclusion on day 2.

Recombinant human IL-2 (rhIL-2) (R&D Systems, Minneapolis, MN.) at 5-500 ng/ml was added to some PHA-stimulated PBMC cultures either 1 hr prior to, at, or 24 hrs after addition of the PHA.

Phorbol 12-myristate 13-acetate (PMA) can bind directly to and activate protein kinase C, leading to DNA synthesis and T-lymphocyte proliferation. Calcium ionophores such as ionomycin can increase the cytosolic calcium concentration of T-cells and lead to T-cell division. PMA and ionomycin act synergistically to stimulate IL-2 production

and the proliferation of T-cells independent of an accessory cell influence. PMA at 10 ng/ml and ionomycin at 100 ng/ml were used to activate triplicate cultures of $10^5$ PBMC. Assays were prepared using either serum-free Opti-Mem medium, or 1:2 hNT supernatant, incubated, pulsed, harvested, and the uptake of $^3$H-TdR determined.

Some hNT neurons were cultured in the presence of 1.2 mg/ml N-g-Monomethyl-L-Arginine (Schweizerhall, Inc., Piscataway, N.J.), an inhibitor of nitric oxide synthase for 3 days and the hNT supernatant analyzed for antiproliferative activity.

Supernatant from other cultures of human NCCIT embryonal carcinoma cells, T98G glioblastoma cells, or THP-1 monocytic leukemia cells (ATCC), maintained in RPMI medium were also analyzed for anti-proliferative activity.

**Cell Cycle Analysis.** PBMC were stimulated with PHA in Opti-Mem medium or 1:2 NT2N-CM, harvested at 48 or 72 hrs, washed, stained with the nucleic acid binding dye propidium iodide (Sigma), and analyzed for DNA content by flow cytometry (fluorescence intensity at 600-650 nm). The proportion of cells in each distinct phase of the cell cycle was calculated with ModFit *LT* 2.0 software (Verity Software House, Topsham, MN).

**Detection and Immunoprecipitation of TGF-β.** hNT supernatant was tested for the presence of TGF-β (R&D Systems), and interleukin-10 by ELISA (Genzyme, Cambridge, MA.), and for prostaglandin-E2α, vasoactive intestinal peptide (Peninsula Labs, Inc., San Carlos, CA.), and α-melanocyte stimulating hormone (Phoenix Pharmaceuticals, Belmont, CA.) by EIA.

To remove TGF-β from hNT supernatant, neutralizing anti-TGF-β mAb 240 at 0.5–10.0 μg/ml (R&D Systems) was added and reacted overnight at 4°C under rotating conditions. An excess of protein G Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) was added, and reacted for 10 hours at 4°C. The mixture was centrifuged at 2000 X g for 10 minutes, and the supernatant tested for anti-proliferative activity. Immunoprecipitation of TGF-β was verified by ELISA. In other experiments, 0.01–10.0 μg/ml neutralizing anti-TGF-β mAb 240 was added directly to PHA-stimulated PBMC assays.

We confirmed that purified TGF-β could suppress T-cell proliferation 40 ± 3.5%, and that mAb 240 could neutralize 53% of the antiproliferative effect of TGF-β using the IL-2- and interleukin-4-dependent helper T-cell line HT-2 (Ho et al., 1987) (data not shown). HT-2 cells were maintained in RPMI medium with 50 μM β-2-mercaptoethanol,

and 10 ng/ml rhIL-2. For assays, HT-2 cells were harvested in log phase, suspended in RPMI medium without rhIL-2 at $2x10^5$ cells/ml, supplemented with 7.5 ng/ml rh-interleukin-4 (R&D Systems), and used to establish triplicate cultures. Some assays contained 1:2 hNT supernatant with or without 5.0-50.0 ng/ml rhIL-2. Cultures were incubated for 48 hours, pulsed with $^3$H-TdR, harvested, and analyzed by β-scintillation counting.

We confirmed that purified TGF-β at concentrations detected in some samples of hNT supernatant (100-1000 pg/ml), could suppress HT-2 T-cell proliferation a mean 40 ± 3.5%, but this was less than the 90 ± 9% suppression mediated by the hNT supernatant (n=3) ($p<0.01$) (data not shown). We also confirmed that 0.1-0.4 ug/ml mAb 240 could neutralize 53% of the antiproliferative effect of 250 pg/ml purified TGF-β on HT-2 cells, reducing it from 40 ± 3.5% to 19 ± 3% suppression (n=3) (data not shown). In contrast, as described below, immunoprecipitation of TGF-β did not reduce the suppressive activity of the hNT supernatant.

**Inhibition of Gangliosides.** HNT neurons were cultured 4 days in the presence of 0.5 or 1.0 μM d, l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (Matreya, Inc., Pleasant Gap, PA.), a potent inhibitor of glucosylceramide synthase and ganglioside shedding (Olshefski and Ladisch, 1998; Felding-Habermann et al., 1990). An aliquot of hNT supernatant was collected after the 4-day exposure. hNT neurons were washed, suspended in fresh Opti Mem medium, and aliquots of hNT supernatant saved at 24, 48, and 72 hrs post-washing. Each aliquot was analyzed for antiproliferative activity.

In other experiments, hNT supernatant gel filtration fractions with antiproliferative activity were incubated with Vibrio cholerae neuraminidase-coated agarose beads (Sigma) for 2 hours to eliminate gangliosides, then evaluated for retained antiproliferative activity.

**Interleukin-2 Expression.** The influence of hNT supernatant on the production of IL-2 was determined by comparing IL-2 levels in the supernatant of PHA-stimulated PBMC cultures containing either Opti-Mem medium or 1:2 hNT supernatant. Expressed levels of IL-2 prior to, and 4, 15, 24, and 48 hours after PHA stimulation were determined by ELISA (R&D Systems).

**Gel Filtration.** hNT supernatant was concentrated using ultrafiltration (YM10, Amicon, Danvers, MA), and fractionated using Sephacryl S-300 HR gel in a 2.5 x 95 cm

equilibrated ... with...

column (Amersham Pharmacia Biotech). Each 4 ml fraction was assessed for protein content at 280 nm. Groups of five fractions were pooled, diluted 1:20, and tested for ability to suppress PHA-induced PBMC proliferation.

**Characterization of the Immunosuppressive Protein:** hNT supernatant, or active fractions were treated with either heat (56°C for 30 minutes), or to pH 2 or pH 11, or mixed with trypsin- or carboxypeptidase A-coated agarose beads (Sigma) each for 60 minutes, and tested for retained suppressive activity in a PHA-stimulated assay.

To determine whether the antiproliferative factor(s) could bind to affinity resins, either 250 mg/ml Heparin-Sepharose CL-B gel, or Blue Sepharose gel (Amersham Pharmacia Biotech) were separately combined with either hNT supernatant or active fractions at ratios of 1:1 to 4:1. Each separate mixture was centrifuged, and the supernatants and washes containing unbound proteins were retained for analysis. The bound protein was eluted from each gel with 0.25M-1.5 M NaCl. Eluted fractions were desalted with PD-10 Desalting Columns (Amersham Pharmacia Biotech). The unbound and eluted fractions were tested for retained suppressive activity in a PHA-stimulated assay.

Active fractions were also tested for binding to Sepharose Fast Flow resins comprised of weak (DEAE) or strong (Q) anion exchangers, or weak (CM) or strong (SP) cation exchangers (Pharmacia Biotech). Bound fractions were removed from exchange resins using 0.25-1.5 M NaCl in 50 mM HEPES buffer. The eluted fractions were dialyzed against 0.15 M NaCl in 0.5 M HEPES. The unbound fraction and eluted fractions were tested for suppression of T-cell proliferation.

The peak active fraction from Sephacryl S-300 gel filtration was used for isoelectric focusing using the Bio-Rad Rotofor system. A broad (pH 3-10) ampholyte range, followed by a narrow (pH 4-6) ampholyte range were used to focus peak active fractions. After focusing with 1.5 ml ampholyte (pH 4-6, 40% w/v) and 45.5 ml doubly distilled $H_2O$ for 60 minutes at 15 watts and 4°C, the peak active fraction was identified in a PHA-stimulated assay. The peak active fraction, 2.1 mg in a 3.0 ml volume, was then focused for an additional 3 hours using the same conditions. Ampholytes were removed by the addition of 0.25 ml of 5 M NaCl per ml, and fractions were exhaustively dialyzed against 50 mM HEPES, pH 7.2, containing 150 mM NaCl for 22 hours at 4°C.

## 3. RESULTS

**hNT Neurons Express MHC class I Proteins.** The amplified products of polymerase chain reactions using Ntera2/D1 genomic DNA as template indicated a MHC class I and II genotype of A1 B8 Bw6 Cw7 DR3 DR52 (Figure 1). The surface expression of MHC molecules on hNT neurons was detected using a complement-dependent cytotoxic technique and limited to the class I proteins A1 B8 Bw6. No surface expression of MHC class II proteins was detected on hNT neurons.

**hNT Neurons Do Not Stimulate PBMC Proliferation.** We first tested the immunogenic potential of hNT neurons *in vitro*, by mixing hNT neurons with allogeneic lymphocytes, and assessing for the DNA synthesis and proliferation of lymphocytes. In control MLCs involving 6 separate donors, responder PBMC proliferated in the presence of unmatched, irradiated stimulator PBMC, with a mean stimulation index of $9.4 \pm 7.9$ (n=16). In spite of the surface expression of MHC class I proteins on hNT neurons, irradiated hNT neurons did not induce responder PBMC to proliferate in MLNC (Figure 2A). hNT neurons were derived from Ntera2/D1 cultures treated with RA, and exposed to either mitotic inhibitors or to 25 Gy $\gamma$-irradiation ($^{137}Cs$) to eliminate non-neuronal cell growth. In either case, hNT neurons did not induce responder PBMC from 4 separate donors to proliferate, with a mean stimulation index of only $0.2 \pm 0.1$ (n=12), significantly less compared to control MLCs ($p<0.01$). Viability of PBMC in MLNC on day 4 was comparable to that of PBMC in control MLC, and routinely >90% in trypan blue dye exclusion assays.

**hNT Supernatant Suppresses Allogeneic MLC.** To determine whether this absence of PBMC proliferation in the presence of hNT neurons was attributable to a soluble factor expressed by hNT neurons, we added supernatant from hNT cultures to allogeneic MLC so that the resultant concentration of hNT supernatant was 1:2. hNT supernatant from cultures maintained with or without serum, and treated earlier with either mitotic inhibitors or $\gamma$-irradiation to eliminate non-neuronal cell growth, suppressed the proliferation of responder PBMC in allogeneic MLCs by more than 98% compared to control MLC ($p<0.01$), with a mean stimulation index of only $0.1 \pm 0.1$ (n=9) (Figure 2A). Viability of PBMC in MLC on day 4, cultured either in control medium or in the presence of 1:2 hNT supernatant, was comparable, and >90% as indicated by trypan blue dye exclusion.

**hNT Supernatant Suppresses T-cell Proliferation and IL-2 Production.** hNT supernatant with or without serum, from Ntera2/D1 differentiated cultures treated earlier

with either mitotic inhibitors or γ-irradiation to eliminate non-neuronal cell growth, significantly suppressed PHA-stimulated proliferation 87 ± 12% (n=20), and concanavalin A-stimulated proliferation 79 ± 19% (n=8) each ($p<0.01$) (Figure 2B). Dilutions of hNT supernatant suppressed PHA-stimulated proliferation in a dose-dependent manner, when tested at 1:2, 1:20, and 1:200 final concentrations, 93%, 62%, and 21%, respectively. That hNT supernatant could also block ongoing T-cell proliferation was demonstrated by adding hNT supernatant up to 48 hrs after PHA stimulation, with a mean suppression of 84 ± 1% (n=6). This suppression of mitogen driven proliferation of PBMC by hNT supernatant was mediated without a reduction in PBMC viability, determined 2 days after PHA stimulation, with >90% cells viable by trypan blue exclusion.

Control PHA-stimulated cultures of PBMC expressed 621 ± 41 pg/ml IL-2, 48 hours after mitogen stimulation. In contrast, PHA-stimulated PBMC cultured in the presence of 1:2 hNT supernatant expressed significantly less IL-2, with mean IL-2 levels detected by ELISA at 48 hours post-stimulation of only 223 ± 111 pg/ml ($p<0.01$) (Figure 3).

Adding supplemental IL-2 to the PHA-stimulated assays did not activate the "quiescent" PBMC cultured in the presence of hNT supernatant. When 5-50 ng/ml rhIL-2 was added 1 hr before PHA stimulation to control assays, T-cell proliferation was increased a mean 47 ± 12% (n=2) above levels induced by PHA alone (Figure 2B). In marked contrast, adding supplemental rhIL-2 did not reverse the T-cell suppressive activity of the hNT supernatant, which persisted in suppressing the PHA-stimulated PBMC cultures a mean 97 ± 3% (n=3) ($p<0.01$) (Figure 2B).

To further assure that hNT supernatant did not lower PBMC viability in immunoassays, propidium iodide stained PBMC were evaluated by flow cytometry, 48 and 72 hrs after PHA-stimulation. Cell cycle analysis revealed that hNT supernatant did not reduce PBMC viability compared to controls (90.8 ± 1.7%), that hNT supernatant held PHA-stimulated PBMC in a growth arrested, $G_0/G_1$ phase (97 ± 2%), and that the proportion of PBMC in either S phase or $G_2$/M phase was reduced by as much as 92% to a mean of only 1.5 ± 0.7% (n=2) (Figure 4). The proportion of PBMC undergoing apoptosis, necrosis, and the amount of cellular debris in the modeled events were not different regardless of treatment.

PHA activation of T-cell proliferation results in cross-linkage of the T-cell receptor-CD3 complex, and is influenced by accessory cell signals. To determine whether hNT supernatant could suppress the direct activation of T-cells by PMA or ionomycin, independent of an accessory cell influence, and independent of TCR-CD3 interactions, hNT supernatants were added to PBMC cultures stimulated with 10 ng/ml PMA, or 100 ng/ml ionomycin, or both. As shown in Figure 5a, hNT supernatant consistently and significantly suppressed the direct activation of T-cell proliferation by PMA, ionomycin, or both 99 ± 1% (n=3) each ($p<0.001$).

The antiproliferative effect of hNT supernatant was not mimicked by the culture supernatant of other embryonal carcinoma (NCCIT), or glioblastoma (T98G), or monocytic leukemia (THP-1) cell lines with each eliciting less than a mean 1.5% suppression of the PHA assay (n=6), data not shown.

hNT neurons were cultured for 4 days in the presence of 0.5 or 1.0 μM d, l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol, a potent inhibitor of glucosylceramide synthase and ganglioside shedding. An aliquot of hNT supernatant was collected after the 4-day exposure. hNT neurons were washed, suspended in fresh serum-free Opti-Mem medium, and aliquots of hNT supernatant saved at 24, 48, and 72 hrs post-washing. In spite of this ganglioside-inhibiting treatment of neuron cultures, the suppressive activity of the hNT supernatant was retained, with 93 ± 10% inhibition of the PHA assay (n=4), data not shown.

We also cultured hNT neurons in the presence of 1.2 mg/ml N-g-Monomethyl-L-Arginine, an inhibitor of nitric oxide synthase for 3 days and found that the suppressive activity of the hNT supernatant was fully retained (data not shown).

We sought, but could not find evidence that the hNT supernatant had T-cell suppressive levels of prostaglandin-E2α (assay threshold of >39 pg/ml), α-melanocyte stimulating hormone (>1.3 ng/ml), vasoactive intestinal peptide (>0.7 ng/ml), or IL-10 (>8.5 pg/ml).

Although TGF-β was detected in 81% of hNT supernatant aliquots at a mean level of 753 ± 512 pg/ml, aliquots of hNT supernatant without detectable TGF-β (threshold >0.1 ng/ml) fully suppressed the PHA assay 94 ± 5% (n=3), data not shown. TGF-β was removed from samples of hNT supernatant using a neutralizing anti-TGF-β mAb and an excess of protein G Sepharose. Complete immunoprecipitation of TGF-β, verified by ELISA, did not reduce the hNT supernatant-mediated suppression of PHA-

stimulated T-cell proliferation, with 97 ± 3% suppression of the PHA assay prior to immunoprecipitation, and 93 ± 1% suppression after TGF-β immunoprecipitation (n=5), data not shown. In other experiments, addition of 0.01–10.0 μg/ml neutralizing anti-TGF-β mAb 240 directly to the PHA-assay did not alter the suppressive activity of the hNT supernatant, with 97 ± 1% suppression of the PHA assay retained with mAb treatment (n=7).

**hNT Neurons Express an Immunosuppressive Protein.** These findings encouraged our efforts to concentrate, characterize, and purify the suppressive activity of hNT supernatant. We used the PHA-activated T-cell proliferation assay as a simple and reliable indicator of T-cell reactivity. As shown in Table 1, hNT supernatant lost most of its suppressive activity when heated, or exposed to low pH 2, or treated with carboxypeptidase A. hNT supernatant exposed to high pH 11, or treated with trypsin retained approximately 48% residual T-cell suppressive activity. The antiproliferative activity of hNT supernatant did not bind to Heparin-Sepharose CL-B gel, with unbound fractions suppressing PHA-stimulated T-cell proliferation a mean 99 ± 0% (n=5).

We concentrated hNT supernatant using YM10 ultrafiltration, and fractionated this concentrate using a Sephacryl S-300 HR gel. Each fraction was assessed for protein content, and pools of five fractions were tested for suppressive activity. Pooled fractions were found to suppress the PHA-stimulated assay over a wide molecular mass range of approximately 40-100 kDa (Figure 6). Peak immunosuppressive active fractions had approximately 7.7 μg/ml of total protein.

The antiproliferative activity of peak fractions of a molecular mass range of 40-100 kDa was not degraded by exposure to Vibrio cholerae neuraminidase-coated agarose beads to eliminate gangliosides (Table 1). In contrast, protease treatments using trypsin or carboxypeptidase eliminated most of the suppressive activity of these peak active fractions (Table 1). Although 79% of the total protein in peak fractions bound to the albumin binding resin Blue Sepharose, the unbound fraction continued to suppress the proliferation of PHA-stimulated T-cells a mean 75 ± 5% (n=2), suggesting that the immunosuppressive protein expressed by hNT neurons was not bound to albumin.

Peak active fractions were also tested for binding to weak or strong anion or cation exchangers. Bound fractions were eluted from exchange resins and dialyzed against HEPES buffer, then bound and unbound fractions were tested for suppressive

activity. The T-cell suppressive activity of 40-100 kDa peak active fractions consistently bound to anion, but not to cation exchanger resins, indicating a net anionic charge. Unbound fractions of weak (CM) and strong (SP) cation exchange resins continued to suppress the PHA-stimulated assay a mean 77 ± 31% and 99 ± 0%, respectively (n=2) (Table 1). In contrast, unbound fractions of weak (DEAE) anion exchange resin suppressed T-cells only 10 ± 17%, and unbound fractions of strong (Q) anion exchange resin had no residual T-cell suppressive activity (n=3). Bound proteins that were eluted from the anion exchangers suppressed the PHA-stimulated assay 99% (data not shown).

We next used the peak active fraction from Sephacryl S-300 gel filtration for isoelectric focusing (IEF). Preliminary broad range isoelectric focusing indicated that the immunosuppressive protein had an isoelectric point of approximately 5. Consequently, we then focused the peak active fraction from gel filtration using a narrow ampholyte pH range of 4-6. Of the twenty fractions collected, only IEF fraction #10 suppressed the proliferation of T-cells more than 70%, indicating that the hNT immunosuppressive protein had an isoelectric point of 4.8 (Figure 7). When either PHA mitogen or PMA/ionomycin were used to activate T-cells, IEF fraction #10 consistently suppressed T-cell proliferation by >70%, each ($p<0.01$) (Figure 5B).

## 4. DISCUSSION

Ntera2/D1-derived hNT neurons are a promising alternative to embryonic neurons for transfection and transplantation therapies to ameliorate the dopaminergic deficit of Parkinson's disease, or Huntington's chorea, or other neuro-deficits and disorders (Trojanowski et al., 1997; Cook et al., 1994), and have been used in Phase I clinical trials involving patients with fixed motor deficit following cerebral stroke (Fackelmann, 1998). In light of the therapeutic potential of hNT neurons, and the paucity of existing data regarding the MHC and immunological features of Ntera2/D1 derivatives, we tested hNT neurons for their potential to regulate immune cells *in vitro*.

Although others have demonstrated using broad, nonspecific antisera that differentiation of Ntera2/D1 cells to hNT neurons results in the expression of some unspecified MHC class I and β-2 microglobulin molecules (Segars et al., 1993), we determined the specific MHC class I and II genotype and phenotype of these cells. In spite of the demonstrated surface expression of MHC class I proteins A1 B8 Bw6, hNT neurons did not activate the proliferation of allogeneic immune cells *in vitro*. Further, we showed that this lack of allogeneic T-lymphocyte activation by hNT neurons *in vitro* was not attributable to low constitutive MHC class I expression on the surface of the hNT neurons, since hNT supernatants potently suppressed T-cell proliferation in a dose-dependent manner.

Herein, we report that hNT neurons express an immunosuppressive protein that has a molecular mass of 40-100 kDa, an isoelectric point 4.8, and a net anionic charge, which does not migrate with albumin, and whose abrogating effect on T-cell activation and on-going T-cell proliferation is direct, not mediated through the TCR-CD3 complex, is not a consequence of altered accessory cell signals, and results in a significant reduction in the level of IL-2 expressed by T-cells. Supplemental IL-2 could not override this suppressive effect, or activate the viable but quiescent T-cells, which had been arrested in the G0/G1 phase of the cell cycle by the hNT supernatant.

Our initial evaluations of the immunosuppressive properties of the hNT supernatant were guided by precedents which showed that retinoic acid treatment of other embryonal carcinoma cell lines can increase shedding of gangliosides (Chen et al., 1989; Osanai et al., 1997), or expression of TGF-β (Rizzino et al., 1983), which can be immunosuppressive *in vitro*.

We developed multiple lines of evidence that dismiss the potential that the hNT immunosuppressive effect could be attributed to gangliosides shed from the hNT

neurons. Inhibition of T-cell proliferation and IL-2 production by ganglioside-enriched supernatants from brain lipid homogenates is found in the lipid-enriched and protein-depleted fraction (Irani et al., 1996; Irani et al., 1997). In contrast, hNT supernatant was used directly in immunoassays, without a selective lipid extraction enriching for gangliosides, and effectively suppressed T-cell proliferation. Pretreating brain ganglioside-enriched supernatant with neuraminidase eliminates the inhibitory effect of the brain-derived supernatant on T-cells (Irani et al, 1997). In contrast, neuraminidase pretreatment of hNT supernatant peak active fractions did not reduce their suppressive effect on T-cell proliferation. Brain tumor cells can shed gangliosides *in vitro* and *in vivo*, potentially in monomeric form (<2 kDa), bound to albumin (68 kDa), or as micelles (130 kDa) (Kong et al., 1998; Valentino et al., 1990). In contrast, the T-cell suppressive protein in hNT supernatant ranged in mass between 40-100 kDa, and did not segregate with albumin or other Blue Sepharose-bound proteins. Tumor cell shedding of gangliosides *in vitro* can be inhibited 83% by culturing cells in the presence of 1.0 μM d, l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol, an inhibitor of glucosylceramide synthase (Felding-Habermann et al., 1990). In contrast, culturing hNT neurons in the presence of 0.5 or 1.0 μM d, l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol did not reduce the suppressive effect of hNT supernatant on T-cell proliferation.

Similarly, several lines of evidence dismiss the potential that TGF-β was responsible for the dramatic suppression of T-cell proliferation by hNT supernatant. hNT supernatant without detectable levels of TGF-β (threshold >0.1 ng/ml) suppressed T-cell proliferation. Immunoprecipitation of TGF-β from hNT supernatant with detectable levels using neutralizing anti-TGF-β mAb did not substantially reduce the T-cell suppressive activity of hNT supernatant. Prior reports have shown that although a 1.0 ng/ml dose of TGF-β could suppress 50% of either a PHA or PMA/ionomycin-stimulated T-cell proliferation when added up to 6 hours after stimulation, this suppressive effect of TGF-β was lost 16 hours after T-cell activation (Ahuja et al., 1993). In contrast, hNT supernatant suppressed 84% of a PHA-driven T-cell proliferation, even up to 48 hours after T-cell stimulation. Further, TGF-β is a heparin-binding protein (McCaffrey et al., 1992), but the hNT immunosuppressive protein did not segregate with heparin-bound proteins. Finally, the peak active fractions of hNT supernatant that profoundly suppressed T-cell proliferation had no detectable TGF-β by ELISA.

We sought, but could find no evidence of other potential co-mediators of the T-cell suppressive effect of the hNT immunosuppressive protein. Although T-cell proliferation *in vitro* may be modulated by neuropeptides (e.g., vasoactive intestinal peptide) (Sun and Ganea, 1993; Nio et al., 1993), or annexin II (Nygaard et al., 1998), or neurotransmitters (e.g., dopamine) or their metabolites (e.g., homovanillic acid), our evidence suggests that the observed T-cell suppressive effect of hNT neurons and their supernatant was attributable to the expressed of a single anionic protein with an isoelectric point of 4.8.

Studies of Ntera2/D1 cells and their hNT neuron derivative may contribute to the development of a transfectable and transplantable neuron with both therapeutic and protective features. If the hNT immunosuppressive protein can modulate immune responsiveness *in vivo*, then hNT neuron grafts may be both therapeutic and self-protective, either alone or as co-grafts with other cells. This cell system may also serve as a model of the neuronal regulation of immune privilege within the CNS.

Whether immune functions in the CNS are fundamentally different from their systemic counterparts, or whether the observed dissimilarities are due to a unique neuronal microenvironment that is responsible for modifying immune function in the CNS is not fully understood. The novel T-lymphocyte suppressive hNT protein reported herein may have broad applications in preventing graft rejection in transplantation settings, in the treatment of autoimmunities, and in the suppression of severe allergic responses. Further, its neuronal origin introduces the possibility that it may represent a novel class of immunomodulators, which contribute to the maintenance of CNS immune privilege.

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**LEGENDS**

Figure 1: Ethidium bromide-stained gel electrophoresis of the amplified products of MHC class I specific polymerase chain reactions using Ntera2/D1 genomic DNA as template revealed products of 630 (A1), 350 (Bw6), 605 and 415 (B8), and 1060 (Cw7) base pairs, indicating a MHC class I genotype of A1 B8 Bw6 Cw7.

Figure 2: (A) hNT neurons did not activate allogeneic PBMC to proliferate in mixed lymphocyte-neuron cultures (MLNC) compared to the proliferation of allogeneic mixed lymphcyte cultures (MLC). When hNT supernatant was added at a 1:2 dilution, the proliferation of allogeneic MLC was suppressed to basal levels comparable to those of unstimulated controls. Values are mean ± SD.
(B) hNT supernatant significantly suppressed the proliferation of PHA-stimulated PBMC. Supplemental IL-2 augmented the PHA induce proliferation of PBMC, but did not rescue the suppressed T-lymphocytes exposed to hNT supernatant. Values are mean ± SD.

Figure 3: Mean ± SD levels of IL-2 expressed by PHA-stimulated PBMC were significantly less when PBMC were cultured in the presence of a 1:2 dilution of hNT supernatant (□) compared to controls (◊) within 48 hours after establishing the PBMC cultures ($p<0.01$).

Figure 4: Flow cytometric evaluation of propidium iodide-stained PBMC, 72 hours after PHA stimulation, and cultured in the presence of 1:2 hNT supernatant, indicated no reduction in PBMC viability. In this representative single parameter histogram, PBMC viability was 90%, with 96% of the cells in $G_0/G_1$, 2% in S-phase, and 2% in $G_2/M$.

Figure 5: (A) The direct stimulation of T-lymphocyte proliferation by either PMA or ionomycin separately, or PMA/ionomycin together synergistically, was significantly suppressed when a 1:2 dilution of hNT supernatant was incorporated in the assays (each, $p<0.001$). Values are mean ± SD.

(B) Peak T-cell suppressive fractions from gel filtration of hNT supernatant underwent isoelectric focusing. Only isoelectric fraction #10 (IEF-10) significantly suppressed the PMA/ionomycin-induced proliferation of PBMC ($p<0.01$). Values are mean $\pm$ SD.

Figure 6: Fractions from Sephacryl S-300 filtration were evaluated for protein content (continuous tracing), and five sequential fractions were each pooled, diluted 1:20, and assessed for an ability to suppress a PHA-stimulated proliferation of T-cells (vertical bars). Suppression of PHA-driven T-cell proliferation was greatest in fractions corresponding to a molecular mass of 40-100 kDa.

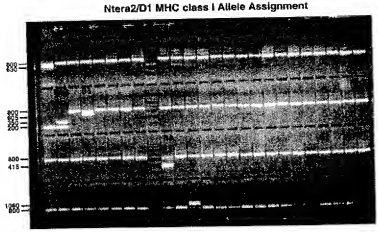
Figure 7: Peak active fractions of hNT supernatant from gel filtration underwent isoelectric focusing using a narrow ampholyte range of pH 4-6 (◊). Isoelectric fractions were assessed for protein content at 280 nm (•). Of the twenty fractions collected and evaluated, only isoelectric fraction #10 (IEF-10) significantly suppressed the PHA-induced proliferation of PBMC (vertical slashed bar) ($p<0.01$). Values are mean $\pm$ SD.

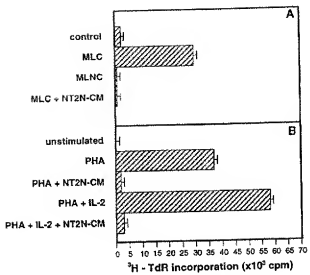
**Table 1. Characteristics of the Immunosuppressive Properties of hNT Supernatant**

| Treatment of hNT Supernatant | Suppression of T-cell Proliferation* |
|---|---|
| None | ++++ |
| 56°C | + |
| PH 2 | + |
| PH 11 | ++ |
| Trypsin | ++ |
| Carboxypeptidase A | + |
| Heparin Sepharose | ++++ |
| Gel Filtration Fraction ~40-100 kDa | ++++ |
| **Treatment of Fraction ~40-100 kDa** | **Suppression of T-cell Proliferation*** |
| Blue Sepharose | +++ |
| Trypsin | + |
| Carboxypeptidase | + |
| Neuraminadase | ++++ |
| Cation Exchange Resin | ++++ |
| Anion Exchange Resin | + |

*% Suppression of PHA-stimulated PBMC proliferation:
++++, 80-100%; +++, 60-79%; ++, 40-59%; +, 10-39%; -, none.

Ntera2/D1 MHC class I Allele Assignment

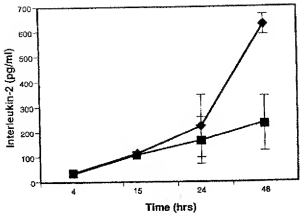





Engelman et. al.,

~~Figure 1~~

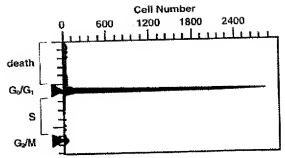
2




Engelman, et. al.,

Figure 3

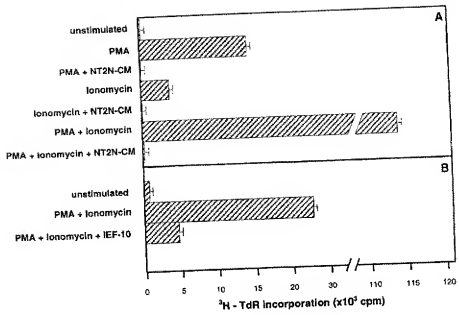
3



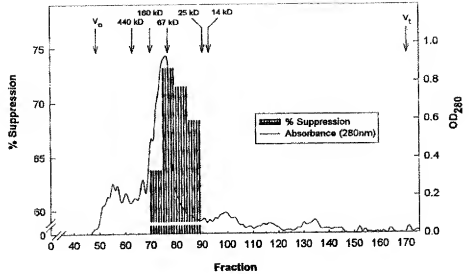

Engelman, et. al.,

~~Figure 2~~

4

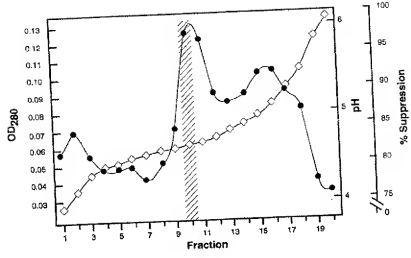

A
unstimulated
PMA
PMA + NT2N-CM
Ionomycin
Ionomycin + NT2N-CM
PMA + Ionomycin
PMA + Ionomycin + NT2N-CM
B
unstimulated
PMA + Ionomycin
PMA + Ionomycin + IEF-10
0
5
10
15
20
30
110
115
120
$^3$H - TdR incorporation (x10$^3$ cpm)

5


160 kD
25 kD
14 kD
$V_o$
440 kD
67 kD
$V_t$
% Suppression
Absorbance (280nm)
% Suppression
$OD_{280}$
Fraction
0
40
50
60
70
80
90
100
110
120
130
140
150
160
170
75
70
65
60
1.0
0.8
0.6
0.4
0.2
0.0

6

FIGURE 4


OD280
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0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
1
3
5
7
9
11
13
15
17
19
Fraction
6
5
4
pH
100
95
90
85
80
75
0
% Suppression

(1)